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TECHNICAL REPORT
EBTNA UTILITY GENE TEST

Genetic testing for lymphatic malformations with or without primary lymphedema

Stefano Paolacci[1], Yeltay Rakhmanov[2], Paolo Enrico Maltese[2]*, Alessandra Zulian[1], Sandro Michelini[3] and Matteo Bertelli[1,2]

Abstract

Lymphatic malformations (LMs) show phenotypic variability, as well as clinical and genetic heterogeneity. Inheritance is autosomal dominant, recessive or X-linked and major genes involved in predisposition for LMs are continuously being discovered. The literature also indicates that somatic mutations play an important role in the development of LMs. In fact, activating somatic mutations in *PIK3CA* have been reported in lymphatic endothelial cells obtained from patients with different kinds of LM. This Utility Gene Test was developed on the basis of an analysis of the literature and existing diagnostic protocols. It is useful for confirming diagnosis, as well as for differential diagnosis, couple risk assessment and access to clinical trials.

Keywords: Primary lymphatic malformations, germline mutations, somatic mutations, EBTNA UTILITY GENE TEST

[1]MAGI Euregio, Bolzano, Italy

[2]MAGI's Lab, Rovereto, Italy

[3]Department of Vascular Rehabilitation, San Giovanni Battista Hospital, Rome, Italy

*Corresponding author: P. E. Maltese
E-mail: paolo.maltese@assomagi.org

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Lymphatic malformations with or without primary lymphedema

(Other synonyms: Primary lymphatic malformations are a group of diseases; see phenotypic variants)

General information about the disease

The term "lymphatic malformations" refers to a broad range of lymphatic system defects (aplasia, hypoplasia and hyperplasia of lymphatic channels and nodes (1), or localized unifocal lesions consisting of dilated lymphatic channels filled with lymph but disconnected from the rest of the lymphatic system (2)). In many cases these defects cause lymphedema (abnormal accumulation of interstitial fluid due to inefficient uptake and reduced lymphatic flow); in other cases lymphatic malformations are not associated with lymphedema. In the past, lymphatic malformations (LMs) and primary lymphedema were considered two different entities, however according to the Hamburg classification, primary lymphedema is a clinical manifestation of LMs appearing in later stages of lymphangiogenesis (truncular LMs) (1), whereas extratruncular lesions, known as cystic/cavernous lymphangiomas, develop during earlier stages of lymphangiogenesis (1). The prevalence of truncular and extratruncular LMs is 1-5/10,000.

In the first step of diagnosis, clinical history and physical examination (3) of patients with LMs should reveal whether the malformation is truncular, extratruncular or syndromic, and if the disorder is inherited or sporadic (4). Lymphoscintigraphy has proved extremely useful for depicting specific lymphatic abnormalities (3). Radioactive colloid is injected into the toe web spaces and uptake by the ilioinguinal nodes is measured at intervals. Lymphoscintigraphy is performed to determine if there is a lack of uptake of radioactive tracer. Other diagnostic tools used to elucidate lymphangiodysplasia/lymphedema syndromes (also in newborns and children) include lymphangioscintigraphy, magnetic

resonance imaging (MR lymphography and MR angiography), computed tomography (CT), CT lymphograms, 3-D oil contrast lymphography, CT-SPECT, ultrasonography, indirect lymphography, near infrared fluorescent imaging (also known as ICG lymphography) and fluorescent microlymphangiography (3). Lymphoscintigraphy is not always essential for diagnosis and one can proceed directly to molecular testing (5).

Differential diagnosis should include hereditary lymphedema; lymphedema-distichiasis; Emberger syndrome; hypotrichosis-lymphedema-telangiectasia syndrome; microcephaly with or without chorioretinopathy, lymphedema and mental retardation; lymphedema-choanal atresia; Hennekam lymphangiectasia-lymphedema syndrome; anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis and lymphedema; congenital lipomatous overgrowth, vascular malformations and epidermal nevi syndrome; and Klippel-Trenaunay syndrome.

LMs are associated with several conditions characterized by allelic and locus heterogeneity and different modes of inheritance. Inheritance can be autosomal dominant, autosomal recessive or X-linked recessive. Genes involved in a predisposition to lymphedema triggered by surgery have also been reported (6, 7).

Autosomal dominant non-syndromic LMs

- hereditary lymphedema 1A (LMPH1A, OMIM disease 153100) - *FLT4* (OMIM gene 136352) (4);
- hereditary lymphedema 1C (LMPH1C, OMIM disease 613480) - *GJC2* (OMIM gene 608803) (8);
- hereditary lymphedema 1D (LMPH1D, OMIM disease 615907) - *VEGFC* (OMIM gene 601528) (9);
- bilateral lymphedema of the lower limbs (OMIM disease not available) - C*ELSR1* (OMIM gene 604523) and *HGF* (OMIM gene 142409) (7, 10).

Autosomal dominant syndromic LMs

- lymphedema-distichiasis (OMIM disease 153400) - *FOXC2* (OMIM gene 602402) (11);
- primary lymphedema with myelodysplasia or Emberger syndrome (OMIM disease 614038) - *GATA2* (OMIM gene 137295) (12);
- hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, OMIM disease 607823) - *SOX18* (OMIM gene 601618) (13);
- microcephaly with or without chorioretinopathy, lymphedema or mental retardation (MCLMR, OMIM disease 152950) - *KIF11* (OMIM gene 148760) (14);
- oculodentodigital dysplasia (ODDD, OMIM disease 164200) - *GJA1* (OMIM gene 121014) (15);
- nonimmune hydrops fetalis and/or atrial septal defect (HFASD, OMIM disease 617300) - *EPHB4* (OMIM gene 600011) (16);
- Noonan syndrome 1, 3, 4, 6, 8 (NS, OMIM disease 163950, 609942, 610733, 613224, 615355) - *PTPN11* (OMIM gene 176876), *KRAS* (OMIM gene 190070), *SOS1* (OMIM gene 182530), *NRAS* (OMIM gene 164790), and *RIT1* (OMIM gene 609591) (17-21);
- Noonan-like syndrome with or without juvenile myelomonocytic leukemia (NSLL, OMIM disease 613563) - *CBL* (OMIM gene 165360) (22);
- Costello syndrome (OMIM disease 218040) - *HRAS* (OMIM gene 190020) (23);
- Noonan-like syndrome with loose anagen hair (NSLH, OMIM disease 607721) - *SHOC2* (OMIM gene 602775) (21);
- cardiofaciocutaneous syndrome 1 (OMIM disease 115150) - *BRAF* (OMIM gene 164757) (24).

Autosomal recessive syndromic LMs

- hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, OMIM disease 607823) - *SOX18* (OMIM gene 601618) (13);
- lymphedema-choanal atresia (OMIM disease 613611) - *PTPN14* (OMIM gene 603155) (25);
- Hennekam lymphangiectasia-lymphedema syndrome 1 and 2 (HKLLS1 and 2, OMIM disease 235510 and 616006) - *CCBE1* (OMIM gene 612753) or *FAT4* (OMIM gene 612411) (26, 27);
- hereditary lymphedema 3 (LMPH3, OMIM disease 616843) - *PIEZO1* (OMIM gene 611184) (28).
- Recently, a new form of HKLLS (HKLLS3), caused by loss-of-function mutations in *ADAMTS3* (OMIM gene 605011), has been described (29).

X-linked recessive syndromic LMs

- anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis and lymphedema (OLEDAID, OMIM disease 300301*)* - *IKBKG* (OMIM gene 300248) (30).

Diseases with paradominant inheritance (as consequence of a second-hit, germline + somatic, variation) associated with LMs

- *RASA1*-related lymphatic malformations (OMIM disease not available) - *RASA1* (OMIM gene 139150) (31, 32);
- congenital chylothorax (OMIM disease 603523) - *ITGA9* (OMIM gene 603963) (33).

Diseases with sporadic onset, due to *de novo* somatic variations, associated with LMs

- *PIK3CA*-associated syndromes (OMIM disease not available) - *PIK3CA* (OMIM gene 171834); the following disorders are included in this spectrum: isolated lymphatic malformations (OMIM disease not available), congenital lipomatous overgrowth, vascular malformations and epidermal nevi syndrome (CLOVES, OMIM disease 612918), Klippel-Trenaunay syndrome (KTS, OMIM disease 149000) and fibro-adipose vascular anomaly (FAVA, OMIM disease not available) (34);
- Proteus syndrome (OMIM disease 176920) caused by somatic mutations in *AKT1* (OMIM gene 164730) (35).

Other likely genes

ADM (OMIM gene 103275), *CALCRL* (OMIM gene 114190), *CDH5* (OMIM gene 601120), *PDPN* (OMIM gene 608863), *RAMP2* (OMIM gene 605154), *NRP2* (OMIM gene 602070), *PROX1* (OMIM gene 601546), *GJA4* (OMIM gene 121012), *CYP26B1* (OMIM gene 605207), *ITGA5* (OMIM gene 135620), *MAP4K4* (OMIM gene 604666), *ASPP1* (OMIM gene 606455), *ARAP3* (OMIM gene 606647), *CDK5* (OMIM gene 123831), *NFATC1* (OMIM gene 600489), *TIE1* (OMIM gene 600222), *ANGPT2* (OMIM gene 601922), *DCHS1* (OMIM gene 603057), *NR2F2* (OMIM gene 107773), *SMARCA4* (OMIM gene 603254), *LPAR4* (OMIM gene 300086), *FOXC1* (OMIM gene 601090), *EMILIN1* (OMIM gene 130660), *RORC* (OMIM gene 602943), *SVEP1* (OMIM gene 611691), *SDC4* (OMIM gene 600017), *PECAM1* (OMIM gene 173445), *VANGL2* (OMIM gene 600533), *FLT1* (OMIM gene 165070), *NOTCH1* (OMIM gene 190198), *RELN* (OMIM gene 600514), *EFNB2* (OMIM gene 600527), *SEMA3A* (OMIM gene 603961), *NRP1* (OMIM gene 602069), *PLXNA1* (OMIM gene 601055), *PROX1* (OMIM gene 601546), *FABP4* (OMIM gene 600434), *SOX17* (OMIM gene 610928), *VCAM1* (OMIM gene 192225), *MET* (OMIM gene 164860), *FOXC2-AS1* (OMIM gene not available), *LZTR1* (OMIM gene 600574), *SOS2* (OMIM gene 601247), *MAP2K2* (OMIM gene 601263), *MAP2K1* (OMIM gene 176872), *PPP1CB* (OMIM gene 600590), *RAF1* (OMIM gene 164760).

Pathogenic variants may include missense, nonsense, splicing, small insertions, small deletions, small indels, gross insertions, duplications and complex rearrangements.

Aims of the test

- To determine the gene defect responsible for the disease;
- To confirm clinical diagnosis;
- To assess the recurrence risk and perform genetic counselling for at-risk/affected individuals.

Test characteristics

Specialist centers/Published guidelines

The test is listed in the Orphanet database and is offered by 22 accredited medical genetic laboratories in the EU, and in the GTR database, offered by 7 accredited medical genetic laboratories in the US.

Guidelines for clinical use of the test are described in disease-specific chapters of Genetics Home Reference (ghr.nlm.nih.gov) and Gene Reviews (36).

Test strategy

Clinically distinguishable syndromes can be analyzed by sequencing only those genes known to be associated with that specific disease using Sanger or Next Generation Sequencing (NGS); if the results are negative, or more generally if clinical signs are ambiguous for diagnosis, a multi-gene NGS panel is used to detect nucleotide variations in coding exons and flanking introns of the above genes.

- If the disorder is familial, the test is performed to identify pathogenic germline variants.
- If the disease is sporadic, the first step is to identify germline variants and check the possibility of a dominant *de novo* mutation.
- If the test is negative or if only a single germline variant in a paradominant gene (*RASA1* or *ITGA9*) is found, the second step is to analyze affected tissues to find *de novo* somatic mutations that could be present only at the site of the malformation.

The test for paradominant (*RASA1* or *ITGA9*) and *de novo* (*AKT1 and PIK3CA*) somatic variations is to compare results obtained from germinal lineage (blood or saliva specimens) and affected tissue. For variant selection, a cut-off value (related to biopsy and blood results) is used, and if the variant frequency is higher than the cut-off value it is considered for further analysis. The cut-off depends on tissue quality, extraction method, biocomputing software and other parameters. Potentially causative variants need to be verified by further means (e.g. cloning + Sanger sequencing, Sanger sequencing, minisequencing).

Genetic analysis should be extended to relatives when the test is positive in familial cases or when a *de novo* germline variant is found that could be inherited by offspring. Potentially causative variants and regions with low coverage are Sanger-sequenced. Sanger sequencing is also used for family segregation studies.

Multiplex Ligation Probe Amplification (MLPA) is used to detect duplications and deletions in *FOXC2*, *GATA2*, *NRAS*, *HRAS* and *BRAF*.

To perform molecular diagnosis, a single sample of biological material is normally sufficient. This may be 1 ml peripheral blood in a sterile tube with 0.5 ml K_3EDTA or 1 ml saliva in a sterile tube with 0.5 ml ethanol 95%. Sampling rarely has to be repeated.

A frozen intra-lesional biopsy specimen, in addition to blood or saliva, is necessary to test for somatic variations.

Gene-disease associations and the interpretation of genetic variants are rapidly developing fields. It is therefore possible that the genes mentioned in this note may change as new scientific data is acquired. It is also possible that genetic variants today defined as of "unknown or uncertain significance" may acquire clinical importance.

Genetic test results

Positive

Identification of pathogenic variants in the above genes confirms the clinical diagnosis and is an indication for family studies.

A pathogenic variant is known to be causative for a given genetic disorder based on previous reports or predicted to be causative based on loss of protein function or expected significant damage to protein or protein/protein interactions. In this way it is possible to obtain a molecular diagnosis in new/other subjects, establish the risk of recurrence in family members and plan preventive and/or therapeutic measures.

Inconclusive

Detection of a variant of unknown or uncertain significance

(*VUS*): a new variation without any evident pathogenic significance or a known variation with insufficient evidence (or with conflicting evidence) to indicate it is *likely benign* or *likely pathogenic* for a given genetic disorder. In these cases, it is advisable to extend testing to the patient's relatives to assess variant segregation and clarify its contribution. In some cases, it could be necessary to perform further examinations/tests or to do a clinical reassessment of pathological signs.

Negative

The absence of variations in the genomic regions investigated does not exclude a clinical diagnosis but suggests the following possibilities:

- Alterations that cannot be identified by sequencing, such as large rearrangements that cause loss (deletion) or gain (duplication) of extended gene fragments.
- Sequence variations in genomic regions not investigated by the test, such as regulatory regions, 5'- and 3'-UTR) and deep intronic regions.
- Variations in other genes not investigated by the present test.

Unexpected

Unexpected results may emerge from the test, for example information regarding consanguinity, absence of family correlation or other genetically-based diseases.

Risk for progeny

If the identified pathogenic variant has autosomal dominant transmission, the probability that an affected carrier transmit the disease variant to his/her children is 50% in any pregnancy, irrespective of the sex of the child conceived.

In autosomal recessive mutations, both parents are usually healthy carriers. In this case, the probability of transmitting the disorder to the offspring is 25% in any pregnancy of the couple, irrespective of the sex of the child. An affected individual generates healthy carrier sons and daughters in all cases, except in pregnancies with a healthy carrier partner. In these cases, the risk of an affected son or daughter is 50%.

In X-linked recessive inheritance, affected males transmit the pathogenic variant to their daughters and the probability that a female carrier transmit the pathogenic variant to her offspring is 50% in any pregnancy irrespective of the sex of the child conceived. Females who inherit the pathogenic variant are carriers and usually unaffected. Males who inherit the pathogenic variant are affected.

De novo somatic variations cannot be inherited or transmitted.

In paradominant inheritance, only the germline genetic variant is transmitted in an autosomal dominant fashion and the probability that carriers transmit the germline pathogenic variant to their children is 50% in any pregnancy, irrespective of the sex of the child conceived.

Limits of the test

The test is limited by current scientific knowledge regarding the genes and diseases.

Analytical sensitivity (proportion of positive tests when the genotype is truly present) **and specificity** (proportion of negative tests when the genotype is not present)

NGS Analytical sensitivity >99.99%, with a minimum coverage of 10X; Analytical specificity 99.99%.

SANGER Analytical sensitivity >99.99%; Analytical specificity 99.99%.

MLPA Analytical sensitivity >99.99%; Analytical specificity 99.99%.

Clinical sensitivity (proportion of positive tests if the disease is present) **and clinical specificity** (proportion of negative tests if the disease is not present)

Clinical sensitivity is estimated at about 25% (2).

Clinical specificity: data not available.

Prescription appropriateness

The genetic test is appropriate when:

a) the patient meets the diagnostic criteria for LMs;

b) the sensitivity of the test is greater than or equal to that of tests described in the literature.

Clinical utility

Clinical management	Utility
Confirmation of clinical diagnosis	Yes
Differential diagnosis	Yes
Couple risk assessment	Yes
Availability of clinical trials can be checked on-line at https://clinicaltrials.gov/	

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